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Microinjection and selection and confirmation of transgenic zebrafish

Chiou-Hwa Yuh

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l-Carnitine ameliorates congenital myopathy in a tropomyosin 3 de novo mutation transgenic zebrafish

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Detailed protocol

1. Amplification of TPM3 gene

I used Human Skeletal Muscle QUCIK-Clone™ cDNA (TaKaRa, Japan) as template and by polymerase chain reaction to amplify the TPM3 gene with TPM3-F (5' ATGGCTGGGATCACCACC 3') and TPM3-R (5' CTACATCTCATTCAGGTCAAGCAG 3') primers by the PCR condition listed below:

Initial Denaturation	94°C for 5 minutes
Denaturation	94°C for 30 seconds
Annealing	56°C for 30 seconds
Extension	72°C for 1 minutes
	Repeat 25 cycles
Final Extension	72°C for 7 minutes

2. Gateway cloning

To generate pME-TPM3, product of TPM3 gene was amplified with the primers attB1-TPM3-F (5 GGGGACAAGTTTGTACAAAAAAGCAGGCTATGGCTGGGATCACCACC 3') and attB2-TPM3-R GGGGACAAGTTTGTACAAGAAAGCTGGGTCTACATCTCATTCAGGTCAAGCAG 3') primers by the PCR condition listed below:

Initial Denaturation	94°C for 5 minutes
Denaturation	94°C for 30 seconds
Annealing	58°C for 30 seconds
Extension	72°C for 1 minutes
	Repeat 25 cycles
Final Extension	72°C for 7 minutes

After PCR amplification, the product was purified by MinElute PCR Purification kit (Qiagen, Germany). Using Gateway system BP reaction (Invitrogen, US) to generate pME-TPM3(WT). The final expression construct pTol2-MLC2:TPM3(WT):pA/CG2 was generated by Gateway system LR reaction (Invitrogen, US using p5E-MLC2, pME-TPM3(WT), p3E-polyA and pDestTol2CG2 vector. After LR recombination reaction, the DNA sample was added into TOP10 competent cells for 30 minutes on ice for the transformation. Transferred the sample to 42 °C water bath for 60 seconds for heat shock transformation. After heat shock transformation, the sample was transferred to ice for 5 minutes immediately. 1 mL of LB broth was added and incubated for 1 hour at 37 °C. Then, bacteria were spread on LB agar containing ampicillin and cultured for 16 to 18 hours at 37 °C. Finally, check the clone by using colony PCR and entrusted DNA Sequencing Core Lab in National Health Research Institute to sequence to confirm the clone sequence. The primers used to confirm the LR recombination reaction colony PCR were listed below:

Primer Name	Sequence (5' to 3')	Size (bp)
MLC2-F2	CCATCACTTTCCCCCTACCT	805
TPM3-R2	CGGTCCAGCTCTTCTTCAAC	
TPM3-F2	AGCCAAGCTGGAAAAGACAA	340
p3E-polyAR	CCCCCTGAACCTGAAACATA	
MLC2-F2	CCATCACTTTCCCCCTACCT	1172
p3E-polyAR	CCCCCTGAACCTGAAACATA	

pME-TPM3(E151A) and pME-TPM3(E151G) were created by site-direct mutagenesis (protocol as below) from pME-TPM3(WT). The final expression construct pTol2-MLC2:TPM3(E151A):pA/CG2 was generated by Gateway system LR reaction (Invitrogen, US) using p5E-MLC2, pME-TPM3(E151A), p3

polyA and pDestTol2CG2 vector. The final expression construct pTol2-MLC2:TPM3(E151G):pA/CG2 was generated by Gateway system LR reaction (Invitrogen, US) using p5E-MLC2, pME-TPM3(E151G), p3E-polyA and pDestTol2CG2 vector.
The sequence of pTol2-MLC2:TPM3(WT):pA/CG2, pTol2-MLC2:TPM3(E151A):pA/CG2 and pTol2-MLC2:TPM3(E151G):pA/CG2 constructs w confirmed.

3. Site-directed mutagenesis

I used QuickChange II site-directed mutagenesis (Agilent Technologies, US) to create the mutation of TPM3 nucleotide 452 mutated from A to C and G. Using TPM3(WT) as template, and TPM3 A452S-F and TPM3 A452S-R as primers, I changed the nucleotide 452 from A to C. Using TPM3(WT) a template, and TPM3 A452G-F and TPM3 A452G-R as primers, I changed the nucleotide 452 from A to G. The sequence of those primers were listed below.

Primer Name	Sequence (5' to 3')
TPM3 A452S-F	CTGAGCTGGCAGSGTCCCGTTGCCG
TPM3 A452S-R	CGGCAACGGGACSCTGCCAGCTCAG
TPM3 A452G-F	CTGAGCTGGCAGGGTCCCGTTGCCG
TPM3 A452G-R	CGGCAACGGGACCCTGCCAGCTCAG

The reaction components and condition was listed below:

Reaction mix

10X reaction buffer 5 µL
DNA template (5-50 ng) X µL
Forward primer (10 µM) 1.25 µL
Reverse primer (10 µM) 1.25 µL
dNTP mix 1 µL
PfuUltra polymerase (2.5 U/µL) 1 µL
Nuclease-free water 40.5-X µL

PCR protocol

Segment 1 95°C for 30 seconds
Segment 2 95°C for 30 seconds
68°C for 1 minute
68°C for 3.5 minutes
Repeat 12 cycles
Segment 3 4°C for 2 minutes

After PCR, 1 µL of *Dpn* I was added and incubated for 1 hour at 37 °C to digestion the amplification products. Then, 1 µL of *Dpn* I -treated DNA sample was added into XL1-Blue supercompetent cells and incubated on ice for 30 minutes. The sample was transferred to 42 °C water bath for 60 seconds for heat shock, then immediately transferred to ice for 5 minutes. 1 mL of LB broth was added and incubated at 37 °C for 1 hour. Then, bacteria were spread on LB agar containing kanamycin and cultured at 37 °C for 16 to 18 hour. Finally, the clones were checked by colony PCR and the DNA was extracted and sent to Sequencing Core Lab in National Health Research Institute for sequencing to confirm the clone.

4. Embryos collection

Embryos can be collected once a week with best results on a regular schedule. The night before embryo collection, adult male and female zebrafish were placed into mating tank with a clapboard after the last feeding. Next morning, the clapboard was removed to start mating. Embryos were collected after 1 hour, removed the dead and unfertilized embryos. Transfer healthy embryos to a clean petri dish containing E3 medium. Keep the embryos in the incubator at 28 °C.

E3 medium:

NaCl (AMRESCO, US) 5 mM
KCl (AMRESCO, US) 0.17 mM
$CaCl_2$(J.T.Baker, US) 0.33 mM
$MgSO_4$(J.T.Baker, US) 0.33 mM

5. Microinjection

Preparation the needle with a micropipette puller, pull a glass capillary into two needles. Inserted the needle into the Nanoject II™ Nanoliter injector (Drummond Scientific, US). For DNA and RNA co-injection, injected 2.3 nL contained 0.05% phenol red into an embryo at one-cell stage. Ensured the embryos did not develop past the two-cell stage. After injection, embryos were placed in E3 medium and incubated at 28°C.

6. Selection and confirmation of transgenic zebrafish

Two days after microinjection, used fluorescent microscope to screen the heart of zebrafish embryo have green fluorescent or not. When the transgenic zebrafish were 3 months old, DNA was extracted by cutting the fins. After amplification the target fragment by polymerase chain reaction, we entrusted DNA Sequencing Core Lab in National Health Research Institute to sequence to confirm the target sequence. The primers used for sequencing were listed in below:

Primer Name	Sequence (5' to 3')	Size (bp)

How to cite:(Readers should cite both the Bio-protocol preprint and the original research article where this protocol was used)

1. Yuh, C. (2022). Microinjection and selection and confirmation of transgenic zebrafish. Bio-protocol Preprint. bio-protocol.org/prep1556.
2. Hsu, P., Wang, H., Tseng, Y., Pan, S., Sampurna, B. P., Jong, Y. and Yuh, C.(2021). l-Carnitine ameliorates congenital myopathy in a tropomyosin 3 de novo mutation transgenic zebrafish. Journal of Biomedical Science 0(0). DOI: 10.1186/s12929-020-00707-1

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